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A Novel Closed-Circular Mitochondrial DNA with Properties of a Replicating Intermediate

(density-gradient centrifugation/electron microscopy/separated strands/ethidium bromide/mouse DNA)

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ABSTRACT A fraction of the covalently closed mitochondrial DNA in mouse L cells contains a replicated heavy-strand segment that is hydrogen bonded to the circular light strand. The inserted single strand is dissociable from the circular duplex at an elevated temperature.

Mitochondrial DNA from animal cells occurs in the form of closed-circular molecules with a molecular weight of about 10×10^6 (1). In the course of a study of the mechanism of replication of this DNA in two strains of mouse L cells, we have found that about one half of the covalently closed molecules in exponentially growing cells contain a short three-stranded DNA region, which we have called a *D-loop*, or *displacement loop* (Fig. 1). The D-loop appears to have been formed in the closed DNA by displacement synthesis of a short progeny strand with a specific region of the mitochondrial light strand serving as a template. This communication presents the results of experiments designed to elucidate the structure and properties of this new kind of closed-circular DNA and considers its role as an intermediate in the replication process.

MATERIALS AND METHODS

The two cell lines studied were the LD line (our designation) obtained from C. Schildkraut, Albert Einstein College of Medicine, New York, and the LA9 line isolated by Littlefield (2) and obtained from L. Crawford, Imperial Cancer Research Fund, London. Mitochondrial DNA (mtDNA) in the LD cells occurs in the form of circular dimers with a contour length of 10 μm. The LA9 cells contain monomeric mtDNA. The spinner-adapted cells were grown in Dulbecco's modification of Eagle's phosphate medium, containing 10% calf serum. The generation times of the LA9 and LD cells were 22 and 20 hr, respectively. Exponentially growing cells, about 2×10^5 cells/ml, were labeled with 0.5 μCi/ml of [^{3}H]thymidine (Schwarz/Mann), 15.6 Ci/mmol, for 3 hr before harvest.

Isolation of crude mitochondria

Labeled cells were harvested at 0–4°C by centrifugation at $1500 \times g$, washed with 0.14 M NaCl–5 mM KCl–7 mM Na_2HPO_4–25 mM Tris (pH 7.5), and resuspended in a 10-fold volume of TE (10 mM Tris–1 mM EDTA)–10 mM NaCl (pH 7.5). After 10 min, the cells were disrupted with 7–10 strokes in a Dounce homogenizer. After addition of 1.5 M sucrose to 0.25 M, about 20 ml of the homogenate was layered onto 15 ml of 1.5 M sucrose containing TE (pH 7.5), and centrifuged for 30 min at 25,000 rpm, 4°C, in a Spinco SW 27 rotor. The crude mitochondrial fraction, withdrawn from the interface, was diluted 6-fold with 10 mM Tris–2 mM $MgCl_2$ (pH 7.5), centrifuged for 15 min at $20,000 \times g$, and resuspended in 0.25 M sucrose–5 mM $MgCl_2$–5 mM NaCl–10 mM Tris (pH 7.2). The mitochondrial suspension was incubated with 25 μg/ml of DNase I and 250 μg/ml of RNase A (both from Sigma Chemical Co.) for 45 min at 37°C. The suspension was chilled to 0°C, EDTA was added to a concentration of 25 mM, and the mitochondria were washed twice by centrifugation with MS buffer (3).

Isolation and purification of mtDNA

MtDNA was extracted from the washed mitochondria, banded in ethidium bromide (EthBr)–CsCl, and purified by velocity sedimentation [ref. 4, Methods *a(i)–(iii)*], except that the incubation with sodium dodecyl sulfate was performed at 37°C. The band velocity sedimentation was performed by layering the sample, after dialysis against 10 × TE–0.1 M NaCl (pH 8.5), onto 4 ml of CsCl (3.5 ml, 1.4 g/ml; 0.5 ml, 1.7 g/ml) containing 100 μg/ml of EthBr (Boots Pure Drug Co. Ltd), and centrifugation for 5 hr at 38,000 rpm, 20°C, in an SW 50.1 rotor. The total mtDNA was collected and banded in EthBr–CsCl at 30,000 rpm, 20°C, for 40 hr in an SW 50.1 rotor. 10-drop fractions were collected from the bottom of the tube and aliquots of the fractions were counted in a scintillation spectrometer (Fig. 2*A*). The DNA samples were dialyzed in the dark against 2 ml of Dowex 50 resin suspended in 25 ml of 10 × TE–0.1 M NaCl (pH 8.5).

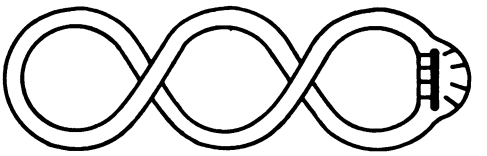

FIG. 1. Diagrammatic representation of closed-circular D-mtDNA containing a displacement loop. The displacing strand is represented by a *heavy line* and the "displaced" strand by a *curved line* with attached bars.

Abbreviations: TE, 10 mM Tris–1 mM EDTA; EthBr, ethidium bromide(3,8-diamino-6-phenyl-5-ethylphenanthridium bromide); mtDNA, mitochondrial DNA; D-mtDNA, mtDNA that consists of molecules containing D-loops; C-mtDNA, mtDNA without D-loops; E-mtDNA, a slow-sedimenting fraction similar to C-mtDNA.

Preparation of the complementary strands of mtDNA

MtDNA isolated from the lower bands in EthBr–CsCl gradients was obtained from LA9 cells labeled for 24 hr with [^{14}C]thymidine (Schwarz/Mann) (0.04 μCi/ml; 50.8 Ci/mol). Unlabeled lower-band mtDNA was added to adjust the specific activity of the DNA to 100 cpm/μg. The mixture was dialyzed against TE–10 mM NaCl (pH 7.5) containing Dowex 50 resin, then into 95% formamide in 1 mM Tris–1 mM EDTA (pH 7.5) at room temperature for 40 min, and finally into TE–10 mM NaCl for 1 hr. The DNA was sedimented through 1.4 g/ml CsCl–100 μg/ml EthBr to remove 7S displacing strands from the closed-circular DNA. The complementary DNA strands were separated in an alkaline CsCl gradient (50 mM K_3PO_4–1.750 g/ml CsCl (pH 12.4) at 30,000 rpm, 20°C, for 60 hr in an SW 39 rotor). 5-drop fractions were collected into 0.4 ml of 20 mM Tris–1 mM EDTA (pH 7.5), and the absorbances at 260 nm were measured (Fig. 2*B*).

Electron microscopy

Samples for electron microscopy were prepared by the formamide–basic protein method (5). In a typical preparation, 2 μl of 1.0 M Tris-base–30 mM disodium EDTA (pH 8.4) and 3–5 μl of H_2O were added to 8 μl of 99% formamide. The solution was chilled to 0°C, and 2–4 μl of DNA in TE, 0.1–0.5 M NaCl (pH 8.0), and 3 μl of cytochrome *c* [1 mg/ml in TE (pH 8.0–8.5)] were added. The solution was layered onto a hypophase of 10% formamide–10 mM Tris–3 mM EDTA (pH 8.4). Films were picked up on parlodion-coated screens and rotary-shadowed with Pt–Pd wire or stained with uranyl acetate (6). Measurements were made from tracings of negatives enlarged on a Nikon 6F projection comparator.

Hybridization of 7S DNA to heavy and light strands

The fractions containing the heavy and light mtDNA strands from alkaline CsCl gradients were appropriately pooled (Fig. 2*B*) and concentrated by evaporation. The 7S single-stranded DNA was obtained from radioactive, closed-circular mtDNA by heat treatment and two sucrose gradient sedimentations. The sample was evaporated to about 0.05 μg/ml (an estimate based on the assumption of complete recovery of 7S DNA). A portion of each of the complements was mixed with 7S DNA so that the final concentrations were 3.0 μg/ml heavy or light strand, 0.01 μg/ml 7S DNA, and 2.0 M CsCl. A control sample contained only 7S DNA in the same solvent.

TABLE 1. *The size of the duplex and single-stranded regions in the D-loops of closed mtDNA from LA9 and LD cells, and the size of 7S DNAs dissociated from the D-mtDNAs*

	(Weight average length/monomer length)100	
	LA9	LD
Duplex region	3.5 ± 0.7 (38)	3.2 ± 0.8 (60)
Single-stranded region	3.5 ± 1.0 (38)	3.2 ± 1.0 (60)
7S single strands	3.1 ± 0.5 (351)	2.3 ± 0.5 (365)

The measured fork-to-fork distances were normalized by the mean length of the circular duplex DNA on the same specimen grid and, in the case of single-stranded DNA, by the contour length of ϕX DNA. The values of 1.7 and 9.6 × 10^6 were taken as the molecular weights of ϕX DNA and mtDNA, respectively. The number of molecules measured is given in parenthesis.

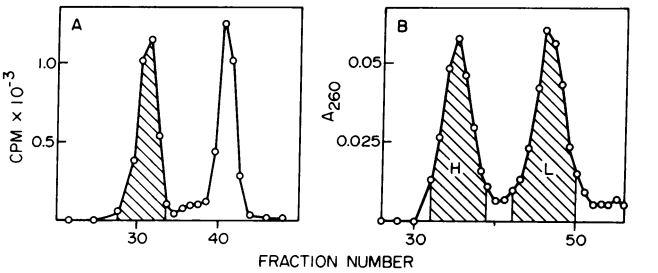

FIG. 2. Buoyant sedimentation patterns. (*A*) total purified mtDNA in an EthBr–CsCl density gradient. (*B*) the separated complements from closed mtDNA in an alkaline CsCl density gradient. The field is directed to the *left*.

The samples were heated at 75°C for 8.5 hr. We calculated (7) that more than 90% of the 7S DNA should have annealed with a mitochondrial complement under these conditions. The hybridized samples were centrifuged in 3.0 ml of CsCl, with a density of 1.710 g/ml, for 72 hr at 30,000 rpm, 20°C, in an SW 50 rotor.

RESULTS

Displacement loops are readily seen in the electron microscope on specimen grids prepared by the formamide–basic film method (Fig. 3). That the D-loop region contains both a duplex segment and a looped-out single-strand DNA segment may be concluded from an inspection of the four examples shown. The single-strand region is thinner and has a less regular contour than the duplex region. It is similar in appearance to the viral ϕX DNA added as a marker. The single-strand region must be DNA rather than RNA, because RNA under these spreading conditions is not fully denatured and collapses with an about 5-fold shortening of the length (8). The lengths of the single-strand and the duplex parts of the D-loop are about equal (Table 1). Moreover, the frequency of D-loops in the purified DNA is unaffected by incubation of the DNA with RNase A (Table 2).

The duplex region, indistinguishable in appearance from the whole circular duplex, is considered to be a DNA–DNA duplex rather than a DNA–RNA hybrid. Chemical experiments presented below show that heating the sample to 90°C in 0.03 M NaCl causes the release of a 7S, alkaline-resistant single-stranded DNA (Fig. 4*F*) with the simultaneous loss of displacement loops.

TABLE 2. *Frequency of D-mtDNA molecules in closed mtDNA isolated from LA9 cells growing exponentially in suspension culture*

Duplex circles (%)	Preparation			
	1	2	2a*	2b†
With D-loops	34	54	51	58
With single-stranded tails	15	1	6	2
Clean	50	45	48	40
Molecules classified	125	207	189	163

* A part of the crude mitochondria used in preparation *2* was processed with omission of the DNase treatment.

† A part of the closed DNA in preparation *2* was treated with RNase before preparation of the specimen grid.

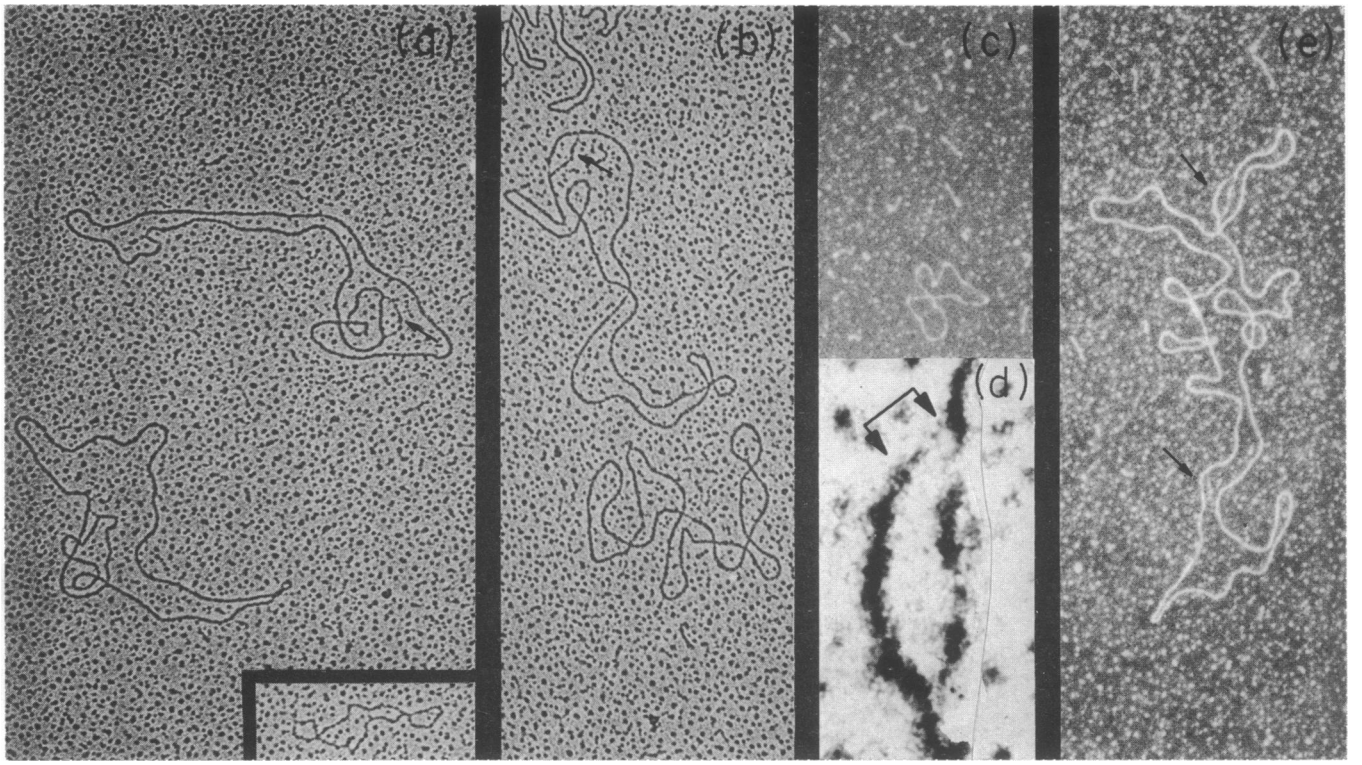

FIG. 3. Electron micrographs of closed-circular mtDNA forms and purified displacing strands. (*a* and *b*) Closed mtDNA from LA9 cells was prepared for electron microscopy by the formamide technique, stained, and shadowed. Displacement loops are indicated by *arrows*. A single-stranded ϕX DNA is present in *a*. (*c*) 7S DNA isolated from a sucrose gradient after sedimentation of heat-treated closed mtDNA from LD cells and prepared for electron microscopy as in *e*. (*d*) The thin region *between the arrows* in an enlargement of a D-loop in the molecule in *e* indicates that the duplex portion of the D-loop appears to be single-stranded near the upper fork. The contrast has been reversed in printing. (*e*) A closed mtDNA from LD cells with two displacement loops. The unshadowed specimen grids were stained and visualized by dark-field electron microscopy.

The relative frequency of molecules containing D-loops (D-mtDNA) in the closed DNA isolated from lower bands in EthBr–CsCl buoyant gradients was 34 and 51% in two separate preparations of mtDNA from LA9 cells (Table 2). Early in this study, we observed that exposure of the closed DNA to low ionic strengths (e.g., 20 mM NaCl) results in a loss of D-loops, presumably by the process of branch migration (9). In this case, progressive pairing of homologous nucleotides in the circular strands displaces the short single-strand. Incomplete branch migration would result in the formation of a circular molecule with a single-stranded tail. If the latter form is taken to be indicative of D-mtDNA, the frequency of D-mtDNA rises to 49 and 56% in the two preparations. Fluorescence photographs of the EthBr–CsCl gradients and the distribution of radioactivity (Fig. 2*A*) indicated that about half of the mtDNA was in the lower band; we may, therefore, conclude that about 25% of the total mtDNA in our preparations contains displacement loops. This surprisingly large frequency in nonsynchronized cells implies that D-mtDNA accumulates and may constitute a "hold point" in the replication process.

The possibility arose that D-mtDNA was formed during the incubation of mitochondria at 37°C with DNase I—the only step in the preparative procedure before lysis in which the temperature rose above 4°C. Half of the partially purified mitochondria obtained in preparation 2 were therefore processed in the normal way, while the other half were lysed directly with SDS. The unchanged frequency of D-mtDNA (Table 2) demonstrates that any DNA synthesis that might occur during the incubation does not contribute significantly to the frequency of D-mtDNA. Incubation of a portion of the purified closed DNA with 5 μg/ml of RNase A had no significant effect on the frequency of D-mtDNA (Table 2).

Fractionation and heat treatment of closed mtDNA

From our knowledge of the effects of superhelical turns on the sedimentation velocity properties of closed duplex DNA, we anticipated that the D-mtDNA, containing a displacing strand with about 3% of the genome length, might have a significantly lower sedimentation velocity than the mature DNA. This proved to be the case, as shown in sedimentation velocity experiments performed in 0.5 M NaCl at 20°C (Fig. 4*A* and *B*). The peak fraction, 23, from the leading 38 S band contained 13% D-mtDNA, as compared with 82 and 60% from selected fractions in the 27 S band (Table 3).

Brief heating of unfractionated closed DNA, as well as dialysis against 90% formamide, resulted in the formation of a new species of DNA with a sedimentation velocity of about 7 S in sucrose gradients containing 0.5 M NaCl. The 7 S component was resistant, as evidenced by acid-precipitable radioactivity, to the action of 0.5 M NaOH for 3 hr at room temperature. To clarify the origin of the 7S DNA, the material obtained upon sedimenting 2 μg of closed mtDNA was pooled as indicated to form the fast and the slow samples (Fig. 4*B*). The separate samples were dialysed into 30 mM salt, heated to 90°C for 30–40 sec, and chilled in ice. The

sedimentation velocity experiments demonstrate that the 7S DNA derives from the slow sample. Dissociation of the displacing strand from the 28S D-mtDNA results in the formation of a closed DNA (C'-mtDNA) with a sedimentation coefficient of 38 S. This is the sedimentation coefficient of the major D-loop-free DNA (C-mtDNA) which, before heating, was present in the fast sample (Fig. 4*C*), but was substantially absent in the slow sample (Fig. 4*D*). A portion of the heated slow sample still sediments as an intermediate band, with a sedimentation coefficient of about 28 S. This band, fraction 27 (Table 3), contains mainly E-mtDNA, a closed duplex with a very low superhelix density. Some single-stranded DNA and 9% D-mtDNA were present.

Size of the D-loops and the thermally dissociated single strands

The duplex and the single-stranded regions measured on enlarged tracings of the electron micrographs appeared to be the same size and to correspond to 3.2–3.5% of the genome size (Table 1). The mean length of 7S single-stranded DNA obtained on heating D-mtDNA from LA9 cells corresponds to 3.1 ± 0.5% of the genome length. The double-strand portion of the D-loop often appears to be thin at one or both of the forks (Fig. 3*D*), as though a portion of the duplex adjacent to the D-loop had been denatured by the spreading forces during specimen preparation. We therefore accept the smaller measured length of the 7S single-stranded DNA as the more reliable estimate of the length of the D-loop. The standard deviation (±18%) of the length measurements is smaller than that expected (±27%) for a homogeneous DNA of this size (5). The mean size corresponds to a molecular weight of 1.5 ± 0.25 × 10^5, and to a molecule with 450 ± 80 nucleotides. The D-loops in the monomer mtDNA from the LA9 cells appear to be somewhat larger than in the dimer mtDNA from the LD cells.

Strand specificity in D-loop formation

To determine whether strand specificity is involved in the formation of the D-loop, the purified 7S displacing strands were self-annealed and were annealed with an excess of the separated heavy and light complements of mtDNA obtained from an alkaline buoyant density gradient (12). The weight-average molecular weights of the separated complements, prepared and heated as in these experiments, have been reported to be about 1 × 10^6 (6).

The 7S DNA upon self-annealing (Fig. 5*C*) formed a band with a variance of 33 [fraction units (f.u.)]^2. The reciprocal of the variance may be taken to be a rough measure of the molecular weight. The band width of the 7S DNA did not change when the annealing was performed with the heavy strands having a variance of 8 (f.u.)^2 (Fig. 5*B*). The variance, however, narrowed to 6 (f.u.)^2 (Fig. 5*A*) when the annealing was performed with the light strands, which, in turn, had a variance of 6 (f.u.)^2. The above results alone show that 7S DNA is largely, if not completely complementary to the light strand. The absence of a detectable shoulder of [^{3}H]-thymidine counts that would have been formed by non-hybridized 7S DNA in the experiment in Fig. 5*A* indicates that at least 85% of the 7S DNA anneals with the light strand. The 7S DNA is, therefore, considered to be a segment of the heavy strand.

TABLE 3. *Frequency of D-loop molecules and other forms of mtDNA obtained upon partial purification and thermal treatment of closed mtDNA from LA9 cells*

	Unheated			Heated		
	4*A**			4*E**	4*F**	
Duplex circles (%)	23†	30	34	18	18	27
With D-loops	13	82	60	0	0	9
With single-stranded tails	1	1	2	0	1	3
Clean	85	18	39	100	99	87
Molecules classified	196	133	164	109	112	79

* Figure no. † Fraction no.

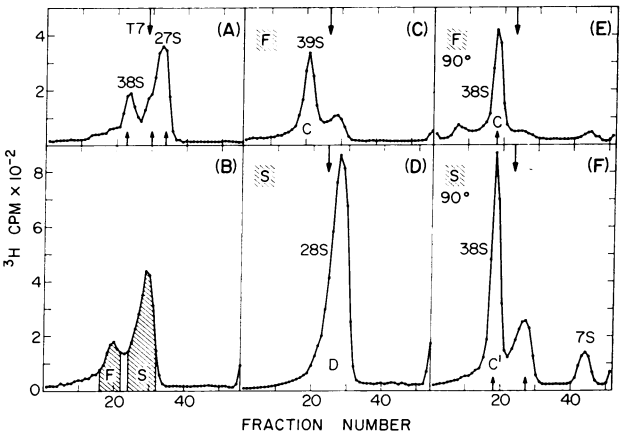

FIG. 4. Sedimentation patterns of LA9 mtDNA from the lower band of an EthBr–CsCl buoyant density gradient (Fig. 2). The DNA was sedimented in 5–20% sucrose gradients containing TE–0.5 M NaCl (pH 8.0) at 20°C for 130 min at 36,000 rpm in an SW 50.1 rotor. The *arrow* ↓ indicates the center of a band of ^{14}C-labeled T7 DNA. The *arrow* ↑ indicates a fraction taken for analysis by electron microscopy (Table 2). Sedimentation proceeds to the *left*. (*A* and *B*), patterns obtained with 1.2-μg and 3-μg samples, respectively. (*C* and *D*), patterns obtained with the fast and slow samples pooled as indicated in (*B*). (*E* and *F*), the fast and slow samples dialyzed against TE–10 mM NaCl (pH 7.5) were heated at 90°C for 40 sec and quenched in ice before centrifugation. **S**, slow sedimenting; **F**, fast sedimenting.

The center of the hybrid band formed in the 7S × L light-annealing experiment is 1-fraction lighter than the center of the band formed principally by the light fragments free of the sequence complementary to the 7S DNA. The 2 mg/ml buoyant shift is the shift expected upon the formation of a duplex between a light-strand fragment with a molecular weight of 1 × 10^6, and 7S DNA with a molecular weight of 1.5 × 10^5.

Superhelix densities of closed mtDNA from LA9 cells

The superhelix densities (superhelical turns/10 base pairs) of the closed mtDNAs separated in the sucrose gradients (Fig. 4) were determined by the buoyant separation method in EthBr–CsCl gradients (Table 4). The superhelix density of the C-mtDNA in the fast sample did not change significantly upon heating. The D-mtDNA, on the other hand, changes in superhelix density upon heating and becomes indistinguishable from C-mtDNA.

The apparent superhelix density calculated from the relative buoyant separation obtained with the slow sample (Fig. 4*B*) was −1.2 × 10^{-2}. The sample contained D-

mtDNA, as well as about 10% E-mtDNA. The position of D-mtDNA in EthBr–CsCl gradients is not expected to be given by the previously derived relation between the buoyant separation and the superhelix density. The duplex region in the D-loop is free to rotate at the forks (Fig. 1) and to bind EthBr without restriction. The displaced strand should bind EthBr with the binding constant of nicked DNA (10). In addition, the single strand might wind about the duplex and permit an increased binding of EthBr at high concentration. These effects decrease the buoyant density and result in an overestimate of the absolute value of the superhelix density.

A second closed mtDNA free of D-loops (E-mtDNA) was also present initially in small amounts in all of the unfractionated closed mtDNA preparations examined in this work. This mtDNA, isolated as the slow-sedimenting component after heating the S sample (Fig. 4B), has a low superhelix density of $-1.0 \pm 0.3 \times 10^{-2}$. The E-mtDNA could have formed from C-mtDNA by the action of a nicking–closing cycle during the isolation procedure. Alternatively, it may be an intermediate in the formation of mature mtDNA.

The buoyant separation experiments provided the further information that we encountered relatively little adventitious nicking while processing the closed DNAs. For example, in the analyses of the C-mtDNA, only 8% of the radioactive label is in the upper band that contains nicked DNA. Similar results were obtained with the other samples analyzed (Table 4).

Unique site for the formation of the displacement loop

It was shown that human dimer mtDNA possesses a head-to-tail arrangement of two monomer genomes (12). The LD line of mouse cells with >99% circular dimer mtDNA was, therefore, examined for its content of D-loops. The fraction of closed-dimer molecules that contain at least one D-loop is again very high, about 42%. Dimers containing two D-loops were also observed at a frequency of about 15%. We would expect that the two D-loops in the dimer molecules would be diametrically opposed if a unique site existed for the initiation of the replicative events that give rise to the D-loops. The two lengths of DNA that separate the D-loops in the dimers were therefore measured. The means and the standard deviations of the shorter and the longer lengths between the forks, expressed as fractions of the total of these lengths, were 0.48 ± 0.03 and 0.51 ± 0.02 in the 25 molecules measured. We conclude that the dimer D-loops are diametrically opposed and that there is, indeed, a unique site on the genome for the formation of the displacement loop.

TABLE 4. *Superhelix densities and sedimentation coefficients of closed mtDNAs from LA9 cells*

Sample	Sedimentation coefficient, svedbergs	Superhelix density $-\sigma_0 \times 10^2$	
		Unheated	Heated
F (4*B*)*	38	2.9	—
S (4*B*)	27	[1.2]	—
C (4*E*)	38	—	2.4
C′ (4*F*)	38	—	2.8
E (4*F*)	28	—	1.0

The superhelix densities were determined by the buoyant separation method (10, 11) in an EthBr–CsCl density gradient. A mixture of closed and nicked SV40 [^{32}P]DNA was added as a marker. The centrifugation conditions were those described by Eason and Vinograd (11). The estimated measuring error is $\pm 0.3 \times 10^{-2}$ units. The number in brackets is to be regarded as an apparent value only. Sample *C* was a pool of fractions 17–20 (Fig. 4*E*). Sample *C′* was a pool of fractions 16–20 (Fig. 4*F*). Sample *E* was a pool of fractions 24–30 (Fig. 4*F*).

* Figure number is given in parentheses.

DISCUSSION

The experiments presented in this communication demonstrate that a substantial fraction of the covalently closed mtDNA isolated from exponentially growing mouse L cells contains a small, unique segment of the heavy strand inserted into the closed duplex. Upon brief heating of the isolated displacement DNA, the (noncovalently) attached heavy-strand segment dissociates and leaves a closed DNA with a sedimentation coefficient and superhelix density indistinguishable from that of the major D-loop-free DNA originally present in the preparation. The physical-chemical results are summarized in functional terms:

$$\text{Displacement DNA} \xrightarrow{\Delta} \text{Parental DNA} + \text{7S Displacing Strand}$$

The displacement loops have been shown in the experiments with dimer DNA from LD cells to occur at a unique site on the circular genome. This result rules out the notion that the D-loops are intermediates in a random repair process. While it is likely that the synthesis of the 7S DNA represents

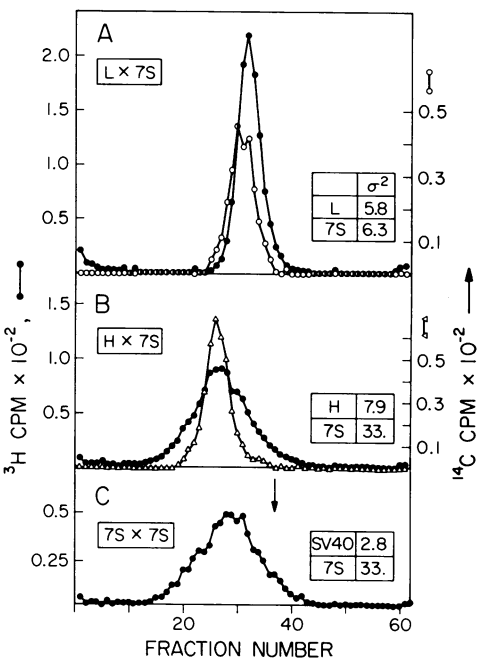

FIG. 5. Strand specificity of the isolated 7S DNA. The 7S [^{3}H] DNA was annealed with the separated light and heavy complements of [^{14}C]mt DNA and was self-annealed as described in the text. The reaction products were centrifuged to equilibrium in a CsCl density gradient. Density increases to the *left*. The variance of the bands, σ^2, was calculated with the relation $\sigma^2 = \Sigma c_i f_i^2 / \Sigma c_i - (\Sigma c_i f_i / \Sigma c_i)^2$, where f and c represent the fraction number and the radioactivity corrected for background, respectively. In a separate experiment, the unannealed 7S DNA formed a band superimposable on the band in *A*. H, heavy; L. light.

the first stage in the formation of progeny molecules, we cannot at present exclude the possibility that D-loops are formed for some other purpose. The fact that larger displacement loops, with various sizes up to that of the genome, have been observed in the mtDNA from the upper band in EthBr–CsCl gradients (unpublished observations) has suggested to us that 7S DNA in nicked parental molecules is, indeed, extended to form the progeny heavy strand.

Among the possibilities for the formation of displacement DNA, we describe here a simple mechanism consistent with the experimental results. The proposed mechanism has two facets. (*a*) Initiation and synthesis of the 450 ± 80 nucleotide heavy strand occurs in closed DNA in the absence of nicking–closing cycles. (*b*) The mature DNA has a zero superhelix density in the cell and acquires its negative superhelix density upon purification and removal of materials that modify the average pitch of the duplex (4). The displacement DNA experiences the same pitch change. A consequence of the above mechanism is that the displacement replication would have to stop when the positive free energy of superhelix formation, ΔG_τ, associated with the topologically required development of a superhelix, is just equal to the negative free energy available from the synthesis reaction. The value ΔG_τ, in cal/mol, of mtDNA is given by Eq. (**24**) in ref. 13, $\Delta G_\tau = 170\tau^2$, where τ is the number of superhelical turns per molecule. The coefficient in Eq. (**24**) has been multiplied by 0.32, the ratio of the molecular weights of simian virus 40 (SV40) DNA to that of mtDNA. From the expression for the derivative $(d\Delta G_\tau/d\tau)$ at $\tau = 45 \pm 8$, we find that 1.5 ± 0.3 kcal/mol is required to unwind the duplex by 36° and to extend the displacing strand by one nucleotide. This free energy is in a reasonable range for the free energy of the synthesis reaction.

Alternative mechanisms for the displacement synthesis, consistent with the results presented here, can be formulated with different, but more complex, coupled assumptions regarding the termination mechanism, the origin of supercoiling, and the occurrence of nicking–closing cycles.

After this work was completed, we were informed by J. ter Schegget and P. Borst that they had interpreted the results of their study (14) of the incorporation of labeled DNA precursors into mtDNA in preparations of chick-liver mitochondria as indicating the formation of a complex of short DNA product that is hydrogen-bonded to closed duplex circles.

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1. Borst, P., and A. M. Kroon, *Int. Rev. Cytol.*, **26**, 108 (1969).
2. Littlefield, J. W., *Nature*, **203**, 1142 (1964).
3. Clayton, D. A., C. A. Smith, J. M. Jordan, M. Teplitz, and J. Vinograd, *Nature*, **220**, 976 (1968).
4. Smith, C. A., J. M. Jordan, and J. Vinograd, *J. Mol. Biol.*, in press.
5. Davis, R. W., M. Simon, and N. Davidson, *Methods Enzymol.*, in press.
6. Robberson, D., Y. Aloni, and G. Attardi, *J. Mol. Biol.*, **55**, 267 (1971).
7. Wetmur, J. G., and N. Davidson, *J. Mol. Biol.*, **31**, 349 (1968).
8. Robberson, D., Y. Aloni, G. Attardi, and N. Davidson, *J. Mol. Biol.*, in press.
9. Lee, C. S., R. W. Davis, and N. Davidson, *J. Mol. Biol.*, **48**, 1 (1970).
10. Bauer, W., and J. Vinograd, *J. Mol. Biol.*, **54**, 281 (1970).
11. Eason, R., and J. Vinograd, *J. Virol.*, **7**, 1 (1971).
12. Clayton, D. A., R. W. Davis, and J. Vinograd, *J. Mol. Biol.*, **47**, 137 (1970).
13. Bauer, W., and J. Vinograd, *J. Mol. Biol.*, **47**, 419 (1970).
14. Ter Scheggert, J., and P. Borst, *Biochim. Biophys. Acta*, in press.